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Award Number: W81XWH-11-1-0549

TITLE: Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer

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Portland, OR 97021-4672

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
15 October 2012	Annual	25 September 2011 – 24 September 2012

4. TITLE AND SUBTITLE
Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0549

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Oregon Health & Science University
Portland, OR 97201-4762

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The focus of the Spellman/Gray work group over the past year has been upon the generation of materials, tools, and data for the purpose of aiding and supporting the research and findings of the entire multi-team collaboration endeavoring to identify antigenic targets of breast cancer-infiltrating T cells. During this time, our group generated the breast cancer cell line lysates necessary to provide uniform sources of major antigens associated with each tumor subtype for stimulation of T cell clones isolated by our collaborators. An analytical pipeline was also developed to perform in silico epitope prediction using RNAseq data as input; the data from which will contribute to the understanding of expressed tumor-associated protein-encoding transcripts. For several of our breast cancer cell lines, MHC-I-bound epitopes were identified via immunoprecipitation and mass spectrometry to assist characterization and prioritization of antigenic targets. Finally, a small molecule drug screen was carried out on breast cancer cell lines and normal T cell clones to determine each drug's potential to combine with T cell-based therapies in promotion of apoptosis via tumor cell signaling pathways and CTL-mediated killing.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

The OHSU Spellman/Gray work group (formerly of Lawrence Berkeley National Laboratory) is one of three collaborators funded by this Department of Defense Breast Cancer Multi-Team Award; the other two being comprised of the Lee work group from City of Hope (formerly of Stanford Medicine Cancer Institute) and the Slansky/Kappler work group from University of Colorado Denver/National Jewish Health. The major objective of this endeavor is to develop novel strategies aimed at the enhancement of the protective effects of anti-tumor T cells *in vivo* in a patient-specific manner based on the hypothesis that partially protective anti-tumor T cells exist within TDLNs in most breast cancer patients. This will be accomplished by identifying the antigens anti-tumor T cells target in different breast cancer subtypes, potentially including antigens preferentially expressed by breast cancer stem cells. We will identify both MHC-I- and MHC-II-restricted antigens driving both CD8 and CD4 anti-tumor T cells *in vivo*, as CD4 T cells are needed to optimally sustain vaccine-elicited CD8 T cells *in vivo* [1]. Identified antigens will be categorized as to breast cancer subtype-specificity or shared status amongst subtypes, with the intention a patient could be matched with an optimal set of vaccine antigens for her tumor. Another novel aspect of this project is the identification of altered peptides (mimotopes) that may more efficiently activate anti-tumor T cells than the natural tumor epitopes. A final objective is to identify small molecule anti-cancer agents that synergize with cytotoxic T lymphocytes (CTLs) to enhance immune-mediated killing. Collectively, this undertaking will produce a set of immunologically validated antigens and mimotopes for major breast cancer subtypes, and a set of agents that cooperate with immune killing. These can be used in combinations in a patient-specific manner to maximize clinical benefit while minimizing toxicity. The tools we develop will enhance the breadth and efficacy of existing and future approaches for immune therapy of breast cancer. We discuss here the Spellman/Gray group's specific efforts toward realizing the goals of this collaboration.

BODY:

Generate tumor lysates pooled from BC cell lines of each major subtype (luminal, HER2+, basal) [Task 4a]

Given the limiting and unpredictable numbers of tumor cells expected to be available from each patient, we produced lysates pooled from multiple breast cancer cell lines of defined subtypes as sources of tumor antigens. We selected 4 to 7 different breast carcinoma cell lines of each subtype (Her2+, claudin-low, basal, and luminal) to produce cell lysates broadly covering the major antigens representative of each subtype. Because these cell lysates will be pulsed on antigen presenting cells, we used the nitrogen decompression bomb technique [2] to protect protein integrity during cell disruption.

According to our calculations, we had to produce at least 400 micrograms of total protein per breast cancer subtype. To produce this amount of protein, we lysed cells from 24-50 150-mm Petri dishes for each cell line over 21 different cell lines. Some cell lines (e.g., SUM159PT, HCC1806) required multiple harvests to achieve a sufficient amount of protein. Cell lines, subtypes, and lysate yields are detailed in Table 1. Each sample has been processed, frozen and shipped to the members of the Lee work group, where lysates will be pooled according to subtype and stored in aliquots for use as a consistent and standard source of breast cancer antigens for stimulation of all T cell lines generated from patients.

Table 1. B**reast carcinoma cell lysates**					
	Cell line	Type	C, mg/ml	V_{Σ}, ml	M_{Σ}, mg
1	SUM159PT	Claudin-low	1.61	2x10	32.2
1	SUM159PT	Claudin-low	2.76	2x9	49.7
2	HCC1806	Basal	1.07	2x7.5	16.1
2	HCC1806	Basal	3.43	2x8.5	58.4
3	SKBR3	Her2+(luminal)	5.48	2x9.5	104.2
3	SKBR3	Her2+(luminal)	5.03	2x9	90.5
4	LY2	Luminal	3.66	2x8	58.6
5	HCC1187	Basal	2.41	2x10	48.2
6	HCC1569	Basal	2.84	2x10	56.9
7	MDA-MB-157	Claudin-low	2.15	2x9.5	40.7
7	MDA-MB-157	Claudin-low	1.56	2x9.5	29.6
8	MCF7	Luminal	4.1	2x9.5	77.9
9	HCC70	Basal	29.2	2x11	642.4
10	JIMT1	Basal	6.0	2x9.5	114.0
11	MDA-MB-231	Claudin-low	5.4	2x10	108.0
12	HCC1500	Basal	6.2	2x10	124
13	ZR75B	Luminal	7.75	2x11	170.5
14	MDA-MB-468	Basal	3.60	2x9	64.8
15	HCC1395	Claudin-low	4.60	2x10	92.0
16	BT549	Claudin-low	3.29	2x10	65.8
17	T47D	Her2+(luminal)	4.17	2x10	83.4
18	CAMA-1	Luminal	6.11	2x10	122.2
19	HCC1419	Luminal	8.40	2x11	184.8
20	BT474	Her2+(luminal)	12.75	2x10	255.0
21	HCC1428	Luminal	4.30	2x10	86.0

Generation and initial analysis of T cell clones [Task 5]

The Spellman/Gray lab is contributing to the progress of this task through identification of MHC-I-restricted epitopes eluted from breast carcinoma cell lines utilizing a combination of immunocytochemistry, immunoprecipitation and mass spectrometry. Our *in vitro* model of breast cancer is a diverse collection of 70 breast cancer cell lines, which are the focus of intensive molecular and phenotypic characterization. We used these breast carcinoma cell lines to determine the sequence and the level of MHC-I-bound epitopes expressed on the cell surface, constructing a comprehensive panel of confirmed epitope sequences. This information will be highly useful to predict and/or support the MHC-I-bound epitopes from breast cancer patients identified by our collaborators via T cell antigen receptor (TCR) sequencing of tumor-reactive T cells or baculovirus-encoded peptide-MHC libraries.

To accomplish this, we first identified MHC-I-positive breast carcinoma cells by staining with MHC-I pan-specific and A2 subtype-specific antibodies. Figure 1 depicts evident cell surface expression of MHC-I A2 molecules in MDA-MB-231, SUM159PT, CAMA-1, and MCF7 cells. As expected, staining with the nonspecific Ms-IgG alone produced a negative result. Figure 2 is a representative example showing quantitative determination of MHC-I cell surface expression amongst different breast carcinoma cell lines.

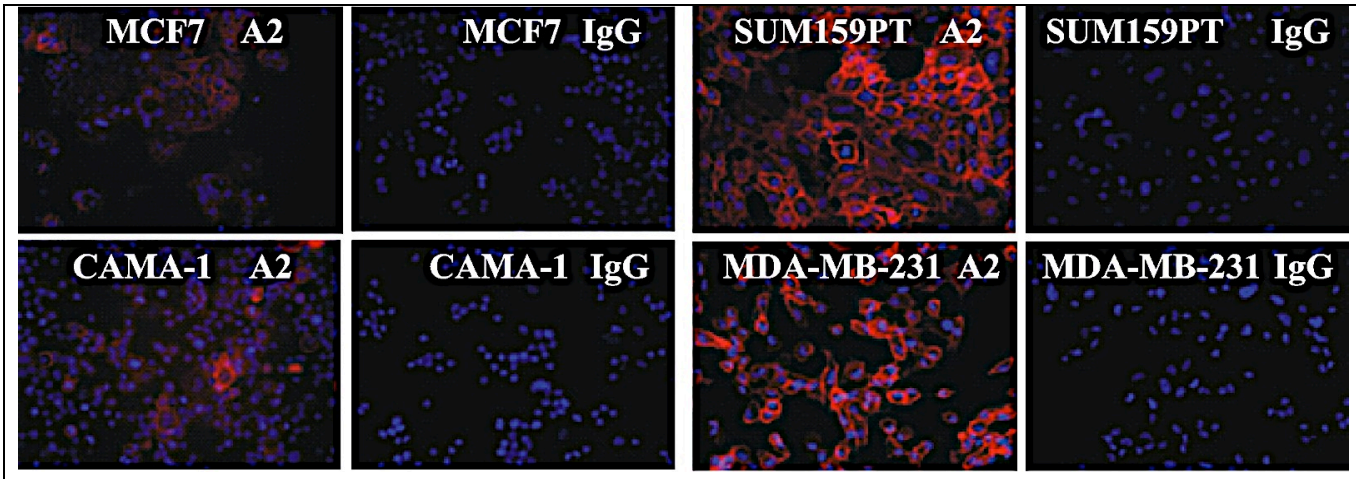

Fig. 1. Immunocytochemistry of MDA-MB-231, SUM159PT, CAMA-1, and MCF7 breast cancer cells with A2-specific and Ms-IgG antibodies.

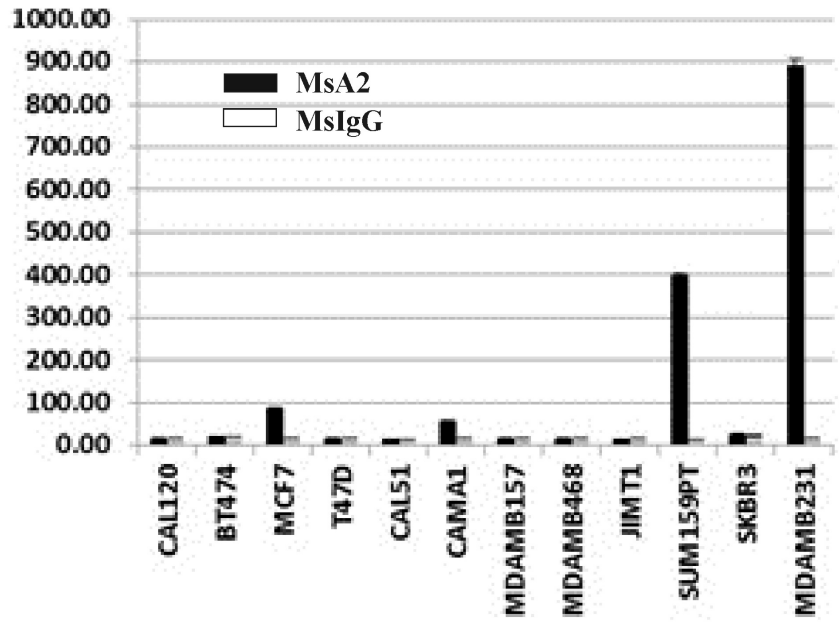

Fig.2. The level of MHC-I A2 cell surface expression. The level of MHC-I A2 cell surface expression was determined by immune staining with A2-specifiic and Ms-IgG antibodies followed by measurement of cumulative signal strength using the Olympus ScanR software.

Once presence/absence of MHC-I was established for each cell line, we developed a very efficient procedure to immunoprecipitate MHC-I molecules followed by elution of MHC-I-bound epitopes with trifluoroacetic acid (TFA). This technique allows us to identify MHC-I-restricted epitopes expressed on the surface of different breast carcinoma cells. Briefly, cells are grown on 12 150-mm Petri dishes to 50-60% confluence. MHC-I expression is induced for 24 hours with INFα and INFγ (103 u/ml, each), then cells are lysed with PBS(50 mM octyl glucopyranoside, aprotinin, leupeptine, pepstatin (1 μg/ml, each), and 1 mM PMSF), 40 ml for 1 h at 4°C. The lysate is cleared via centrifugation at 15,000x, 10 min. and mixed with Ms-MHC I or MsIgG antibodies (100 μg, each) and 100 μl protein G-plus agarose. Following overnight incubation at 4°C, the immune complexes are washed, and MHC-I-bound epitopes are eluted with 1% TFA. The eluate is passed through a 0.2 μM filter units followed by a 10K filter unit to remove agarose beads and high molecular weight proteins. Finally, the eluted peptides are collected and

concentrated using SpeedVac and analyzed by mass spectrometry, thus acquiring the sequence of peptides bound to MHC-I. The results of this experiment are summarized in Table 3, which displays the number of unique peptides identified in each sample, the number of unique proteins represented by those peptides, and the corresponding false positive rates. Results for MDA-MB-231 are provided for three iterations of the experiment, each with sequentially increasing MHC-I-bound epitope yield as the technique was optimized. Further analysis of these results will be carried out to confirm reproducibility of outcomes based on the number of unique versus repeat peptides identified from each replication.

Table 3. MS MHC I-restricted epitopes expressed in breast carcinoma cells.

	Cell line	Subtype	HLA-A2 phenotype	№ peptides	False positive rate, %	№ proteins	False positive rate, %
1	SUM159PT	Claudin-low	positive	**443**	13	**373**	13
2	MDA-MB-231	Claudin-low	positive	**25**	10	**23**	11
2	MDA-MB-231	Claudin-low	positive	**55**	6	**38**	20
2	MDA-MB-231	Claudin-low	positive	**77**	15	**95**	15
3	HCC1187	Basal	positive	**713**	6	**585**	9
4	HCC1569	Basal	negative	**216**	6	**204**	9
5	LY2	Luminal	positive	**306**	5	**261**	11
6	HCC70	Basal	negative	**285**	?	**253**	?
7	HCC1500	Basal	positive	**50**	8	**43**	9
8	MCF7	Luminal	positive	**233**	6	**206**	9
9	HCC1806	Basal	negative	**311**	6	**272**	9
10	HCC1395	Claudin-low	negative	**90**	9	**87**	10
11	MDA-MB-468	Basal	negative	**302**	6	**269**	7
12	BT549	Claudin-low	positive	**122**	1	**50**	20
13	CAMA-1	Luminal	positive	**144**	1	**113**	4
14	T47D HER2+	Luminal	negative	**107**	1	**84**	9
15	MCF12A	Basal	positive	**119**	1	**95**	4
16	CAL-120	Basal	negative	**18**	1	**7**	11
17	HCC1419	Luminal	positive	**38**	2	**23**	10
18	HCC1428	Luminal	positive	**76**	1	**51**	7
19	SUM185PE	Luminal	negative	**106**	2	**91**	2
20	UACC812	Luminal	positive	**122**	2	**93**	3
	Total			**3958**		**3316**	

RNAseq analysis of tumor cells [Task 7]

The molecular characteristics of tumor cells from each enrolled patient will be analyzed by RNAseq for the purpose of ranking epitopes for further development by identifying epitopes which are (1) well expressed in tumors, (2) preferentially or uniquely expressed in tumors, and (3) show broad expression within tumors or tumor subtypes. Additional RNAseq datasets will be utilized to support enrolled patient data and conduct a systematic computational analysis to identify somatic mutations, genomic alterations, and/or transcriptional/post-transcriptional mechanisms resulting in potential breast cancer antigens. These include breast cancer cell lines from our Stand Up 2 Cancer collection as well as greater than 2000 breast cancers available through The Cancer Genome Atlas (TCGA) and International Cancer Genomics Consortium (ICGC). During the interim in which patients are being consented into the project and samples are being collected, the Spellman/Gray computational group has focused attention on development of an epitope prediction pipeline, utilizing breast cancer and normal tissue datasets already available from TCGA, EBI, and GEO.

Our approach to handling the RNA expression data for identification of breast cancer epitopes originally included implementation of the Bowtie/Tophat/Cufflinks packages [3–5] for sequence assembly, quantitation of transcript structures, and determination of differential expression. Due to the finding that biological replicates in RNAseq demonstrate considerable variation as compared to technical replicates and are therefore unsuitable for the Poisson distribution model employed by Cufflinks [6], we have chosen instead to substitute the Myrna software package [6] for detection of differential expression within this large dataset. For our calculation of gene expression level, we will use the modified count normalization scheme that excludes the most abundant transcripts along with the per lane upper quartile of expressed genes for cross-sample normalization [7]. The relationship of the counts (number of reads overlapping a specific gene within a sample) to outcome (disease state or cancer subtype) is then determined by fitting these values to a generalized linear model [6].

The amino acid sequence of differentially expressed transcripts will be interrogated utilizing the collection of epitope prediction tools and analysis resources provided by the Immune Epitope Database (IEDB)[8] to predict the likelihood of MHC-presented epitopes. The IEDB provides downloadable command-line driven tools for the prediction of input sequence binding affinity to MHC class I or class II molecules, thus addressing the high-throughput nature of this project and establishing a preliminary epitope list. Next, we will infer which peptides have the potential to actually become epitopes for T cell recognition by evaluating the flanking regions of each epitope (~30 residues total) for the presence of proper antigen processing structures. Specifically, intact C-terminal proteasomal cleavage sites are necessary for proper release of the epitope from its peptide, and the transporter associated with antigen processing (TAP) must be functional to ensure escape of the peptide from the cytosol into the endoplasmic reticulum. The integrity of both structures will be assessed utilizing the processing prediction tools provided by the IEDB, which combines the predictors of proteasomal processing, TAP transport, and MHC binding affinity and reports the overall potential of the peptide becoming an epitope.

Expression frequencies of putative epitopes in breast tumor tissue, normal tissues, and cell lines will be reported along with statistical evidence of any correlation to breast cancer subtype (e.g., basal, luminal, etc) and/or common mutations (e.g., BRCA1, BRCA2, P13Kα, PTEN, p53).

Of note, we anticipate a limitation on sample availability from enrolled patients due to small tumor size. In this event, priority will be given to T cell isolation and expansion, and RNAseq will only be completed when sample size permits.

Identify small molecule agents that enhance tumor cell apoptosis and enhance CTL killing [Task 12]

As outlined in Aim 4 of the proposal, clinical efficacy of T cell-based therapies will be enhanced in combination with agents promoting tumor cell apoptosis. Support for this idea recently has been published showing that chemotherapy can synergize with CTL-mediated killing [9]; however, chemotherapeutic agents can also inhibit T cell function. In order to identify drugs nontoxic to normal cells, we designed and ran cytotoxicity assays using three normal T cell clones from breast cancer patients and a collection of FDA-approved drugs consisting of 63 compounds. Two additional drugs, MLS0092727 and NSC130362 (lines 65 and 64 of Table 2 respectively), were also tested. MLS00927272 is triazine-based compound found to potentiate TRAIL-induced apoptosis of cancer cells [10], and NSC130362 is a nontoxic derivative of MLS009272. The degree of T cell cytotoxicity was determined by the percent change in ATP present in cell lysate. All assays were done in triplicate. Table 2 displays the drugs tested along with their corresponding averaged IC50 against a set of 45 breast carcinoma cell lines and their cytotoxic effect induced in three normal T cell clones at this IC50. Results in red represent a cytotoxic effect of the drug on T cells, while green indicates a healthy, and perhaps thriving, T cell population despite presence of drug. Imminent Coulter and/or hemocytometer counts will be performed to confirm the results presented in Table 2.

Table 2. Effect of small molecule agents on breast carcinoma and normal T cells.					
	Drug	IC50 (breast carcinoma cells)	Cytotoxicity induced by IC50 in T cells, %		
			476.101	517.7	520.62
1	17-AAG	83 nm	-17	-54	-32
2	5-FdUR	130 μM	40	12	29
3	5-FU	140 μM	33	-1	14
4	AG1478	27 μM	5	-26	-16
5	AS-252424	16 μM	-4	-12	-9
6	CPT-11	10 μM	48	35	38
7	Docetaxel	9 nM	53	37	40
8	Doxorubicin	279 nM	64	49	69
9	Epirubicin	317 nM	29	16	35
10	Etoposide	4 μM	60	43	48
11	Fascaplysin	167 nM	22	-19	-14
12	Ibandronate	63 μM	-34	-56	-52
13	ICRF-193	11 μM	-23	-24	-37
14	Lestaurtinib	638 nM	26	16	12
15	Methotrexate	20 μM	53	45	43
16	NSC 663284	2.3 μM	-10	-4	-1
17	Oxaliplatin	8 μM	16	14	7
18	Oxamflatin	887 nM	35	18	12
19	PD 98059	41 μM	9	-5	-16
20	Purvalanol A	61 μM	59	59	16
21	SB-3CT	70 μM	61	8	43
22	Sigma AKT1-2 inhibitor	3 μM	21	2	0
23	Sunitinib Malate	6 μM	31	10	4
24	Temsirolimus	556 nM	23	6	-4
25	Topotecan	144 nM	72	66	46
26	TPCA-1	13 μM	100	95	99
27	Trichostatin A	9 μM	100	100	100
28	Triciribine	3 μM	37	16	34
29	Vinorelbine	28 nM	68	59	47
30	Vorinostat	76 μM	100	100	97
31	XRP44X	2 μM	15	6	9
32	Bosutinib	3 μM	46	21	34
33	Erlotinib	21 μM	3	-8	-6
34	Glycyl-H-1152	13 μM	-23	-19	-18
35	L-779450	20 μM	1	6	11
36	LBH589	117 nM	98	88	89
37	Pemetrexed	580 μM	72	53	63
38	Nutlin 3a	22 μM	78	40	71
39	Sorafenib	66 μM	100	29	73
40	TCS 2312	560 nM	67	37	55
41	ZM 447439	8 μM	-22	-23	-44
42	TGX-221	11 μM	-29	-27	-45
43	AG1024	21 μM	-25	-26	-34
44	Geldanamycin	29 nM	60	31	43
45	NU6102	20 μM	30	13	8

46	AZD6244	23 μM	-9	-13	-18
47	Ispinesib	46 nM	58	51	47
48	TCS JNK 5a	8 μM	73	73	70
49	Gefitinib	8 μM	8	2	-17
50	BEZ235	12 μM	42	34	26
51	BIBW2992	359 nM	-6	-10	-9
52	Bortezomib	14 nM	17	5	27
53	Carboplatin	47 μM	62	30	50
54	Cisplatin	10 μM	-2	-16	-16
55	Gemcitabine	196 nM	69	40	47
56	Lapatinib	6 μM	-14	-20	-17
57	Paclitaxel	16 nM	51	44	24
58	PD173074	6 μM	-42	3	-13
59	Rapamycin	144 nM	-12	-16	-15
60	Tamoxifen	44 μM	5	-24	-7
61	VX-680	3 μM	-37	-39	-55
63	GSK1120212	2 μM	-64	-47	-81
64	NSC130362	10 μM	-66	-30	-86
65	MLS0092727	10 μM	-43	11	-32

KEY RESEARCH ACCOMPLISHMENTS:

- Generated breast cancer cell line lysates to be pooled according to subtype and ultimately used as a consistent and standard source of breast cancer antigens for all T cell lines to be generated from patients.
- Developed an efficient procedure of MHC-I immunoprecipitation and mass spectrometry to identify MHC-I bound epitope sequences.
- Created an analytical pipeline for *in silico* prediction of novel breast cancer epitopes using RNAseq data as input.
- Evaluated the cytotoxic effect of 65 small molecule agents on a collection of 45 breast cancer cell lines and three normal T cell clones.

REPORTABLE OUTCOMES:

- **NBCC/Artemis Project:** We have applied for and been awarded funding by the Artemis Project®, which was launched by the National Breast Cancer Coalition (NBCC) in September 2010 as a strategic campaign to end breast cancer by the end of the decade. The ultimate goal of the Artemis Project® is to help open the door to personalized breast cancer immunotherapy and promote development of a preventative vaccination for breast cancer. Our proposed project seeks to develop a robust portfolio of native and non-native (e.g., endogenous retroviral/proviral sequences, DNA viruses, microbial genes) antigens across the major breast cancer subtypes using strictly computational means. Data gathered from our DoD project will be leveraged toward the construction and support of an epitope characterization database.
- **MAGE-TAB Sample Archive:** Utilizing a variation upon the MAGE-TAB (MicroArray Gene Expression Tabular) method of spreadsheet-based information tracking [11], the Spellman group collects and maintains an archive of all samples and data generated over the course of this project by all collaborative members. If deemed necessary in the future, this archive can be used to populate a comprehensive, user-friendly database. In the meantime, the archive will have controlled access to all members of the team to protect patient information and data integrity.

CONCLUSION:

The focus of the Spellman/Gray work group over the past year has been upon the generation of materials, tools, and data for the purpose of aiding and supporting the research and findings of the entire multi-team collaboration endeavoring to identify antigenic targets of breast cancer-infiltrating T cells. The breast cancer cell line lysates produced by our group will play a critical role throughout the remaining course of the project as they provide uniform sources of major antigens broadly covering each breast cancer subtype. These antigen pools ultimately will be pulsed onto autologous EBV-transformed B cells to assess tumor reactivity of T cell clones isolated from breast tumors and tumor draining lymph nodes. The RNAseq analysis tool will provide proof of concept of *in silico* epitope discovery from RNAseq data in addition to aiding in the definition of the protein-epitope relationship by (1) enlarging the knowledge base of protein-encoding transcripts beyond the protein models existing in public databases and by (2) restricting the analyses to only the expressed transcripts. The results produced by this pipeline along with the breast cancer cell line MHC-I-bound epitopes already identified by mass spectrometry will be used to rank epitopes for further characterization and development as therapeutic targets. Finally, results of the small molecule drug screen will be confirmed and subsequently evaluated as to their ability of being combined with T cell-based therapies to promote apoptosis via tumor cell signaling pathways as well as CTL-mediated killing.

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APPENDICES:

No appendices to report.